

Non-random chromosome arrangement in triploid endosperm nuclei

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Abstract The endosperm is at the center of successful seed formation in flowering plants. Being itself a product of fertilization, it is devoted to nourish the developing embryo and typically possesses a triploid genome consisting of two maternal and one paternal genome complement. Interestingly, endosperm development is controlled by epigenetic mechanisms conferring parent-of-origin-dependent effects that influence seed development. In the model plant *Arabidopsis thaliana*, we have previously described an endosperm-specific heterochromatin fraction, which increases with higher maternal, but not paternal, genome dosage. Here, we report a detailed analysis of chromosomal arrangement and association frequency in endosperm nuclei. We found that centromeric FISH signals in isolated nuclei show a planar alignment that may result from a semi-rigid, connective structure between chromosomes. Importantly, we found frequent pairwise association of centromeres, chromosomal segments, and entire arms of chromosomes in 3C endosperm nuclei. These associations

deviate from random expectations predicted by numerical simulations. Therefore, we suggest a non-random chromosomal organization in the triploid nuclei of *Arabidopsis* endosperm. This contrasts with the prevailing random arrangement of chromosome territories in somatic nuclei. Based on observations on a series of nuclei with varying parental genome ratios, we propose a model where chromosomes associate pairwise involving one maternal and one paternal complement. The functional implications of this predicted chromosomal arrangement are discussed.

Keywords Endosperm · Chromosome association · Parent-of-origin effect · *Arabidopsis* · Chromocenter · Centromere

Introduction

Eukaryotic genomes are composed of discrete genetic units, the chromosomes, which display a territorial organization in interphase nuclei (Cremer and Cremer 2010). Chromosome territories (CTs) can be visualized by chromosome painting, a technique involving fluorescent in situ hybridization (FISH) with DNA probes covering a specific chromosome or chromosomal region. CTs are present in both plant and animal nuclei but show some specific differences (van Driel and Fransz 2004). The arrangement of CTs is likely influenced by geometrical constraints in the nucleus, biophysical properties of the chromatin polymer, and also by the amount and organization of DNA repeats that mediate heterochromatin formation (Cremer and Cremer 2010; Rosa and Everaers 2008; Schubert et al. 2012). Yet, these constraints are often insufficient to explain CT configurations, for instance in animal nuclei where the arrangement of CTs clearly deviates from predicted random positioning simulated by different

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spatial models. Typically, human nuclei harbor a radial arrangement of CTs, correlating with chromosomal gene density, whereby gene-rich chromosome arms locate preferentially in the nuclear interior (Cremer and Cremer 2010). Remarkably, this arrangement is evolutionary conserved among mammals (primates, rodents, and cattle) and birds (Tanabe et al. 2002). While this observation suggests a functional role for this organizing principle, it also argues for epigenetic instructions overriding geometric and biophysical constraints. By contrast, no radial arrangement has been reported in insect nuclei. Instead, in *Drosophila*, somatic pairing between homologous CTs is prevalent (McKee 2004). In flowering plants, the situation is different again. In species with large metacentric chromosomes, the so-called Rabl configuration—with centromeres (CENs) clustered at one and telomeres (TELs) at the opposite nuclear pole—is maintained in several cell types, reflecting chromosome positioning in anaphase (Schubert and Shaw 2011).

In *Arabidopsis*, a plant species with a genome size and chromosome number similar to that of *Drosophila*, CTs adopt a random configuration and homologous association is the exception rather than the rule. Specifically, the association frequencies (3–6 %) of CTs is not different from those computed with a model of 1-Mb spherical chromatin domains (SCDs), simulating random configuration (Pecinka et al. 2004). Random CT organization was detected in nuclei of differentiated (leaf, root) and dividing (meristematic) tissues of both *Arabidopsis thaliana* and *Arabidopsis lyrata*, the latter having a higher DNA content and chromosome number (reviewed in Schubert and Shaw 2011; Schubert et al. 2012). At a scale of ~100 kb, the association frequency of homologous loci also follows a random spatial distribution (RSD) model (Pecinka et al. 2004). Recently, chromosome configuration capture-based analyses (4C and Hi-C) of interacting domains confirmed a global chromatin folding according to a fractal globule model without a preferential association between homologous chromosomes (Feng et al. 2014; Grob et al. 2013, 2014; Wang et al. 2015). What emerged from these studies is that domains of low or high interaction frequency, i.e., loose or compacted structural domains, form locally on chromosome arms. These domains correlate with well-defined epigenetic indexing of chromatin, corresponding to distinct functional states with regard to, e.g., transcriptional competence, but are independent of CT organization (Feng et al. 2014; Grob et al. 2013, 2014; Wang et al. 2015).

Collectively, these cytogenetic and molecular studies support the view that *Arabidopsis* nuclear genome organization follows geometrical and biophysical constraints but is otherwise random (Del Prete et al. 2014; Schubert et al. 2012), at least in somatic tissues.

Here, we report that the nuclear organization of the triploid endosperm genome of *Arabidopsis* is not random, deviating from the situation in diploid somatic nuclei. The endosperm, an extra-embryonic tissue of the seed, is the product of

fertilization of the homo-diploid central cell by a haploid sperm cell. Hence, the endosperm genome consists of two maternal and one paternal chromosome complement. This triploid genome has a peculiar chromatin organization, characterized by a specific heterochromatin fraction that is interspersed within euchromatin and modulated by the dosage of parental genomes (Baroux et al. 2007). We used FISH to study the organization of endosperm nuclei in more detail. We found an unusual planar alignment of centromeric FISH signals in three-dimensional (3D) reconstructed images of flow-sorted nuclei. Importantly, quantification of FISH signals combined with computed random simulations showed a preferential pairwise association of centromeres, chromosomal segments, and entire chromosome arms. While the parental origin of the associated domains remains to be resolved, the number of chromocenters (CCs) was found to be dependent on the maternal dosage and thus suggests a preferential interaction between maternal and paternal chromosomes. These observations raise the question whether chromosomal interactions underlie epigenetic regulation, leading to dosage-dependent and parent-of-origin-specific gene expression in the endosperm.

Results

Geometrical characteristics of flow-sorted seed nuclei

To compare chromosome organization in endosperm nuclei with that in nuclei from the surrounding tissues, we flow-sorted seed nuclei according to their DNA content as described (Baroux et al. 2007). We isolated 2C and 4C fractions as well as endosperm-specific 3C and 6C fractions from diploid plants and 6C and 12C endosperm nuclei from tetraploid plants of the same accession. The 4C, 6C, and 12C fractions correspond to nuclei with a replicated genome either in the course of a regular cell cycle or, most likely, as a result of endoreduplication (Larkins et al. 2001). Measurements of nuclear volume normalized for the ploidy confirmed that endosperm nuclei harbor a less condensed chromatin compared to somatic seed nuclei (Online Resource 1), similar to what was previously reported in whole-mount seed tissues (Baroux et al. 2007). This finding indicates that the isolation procedure preserves global nuclear architecture.

To analyze chromosome organization, we employed FISH against specific genomic regions or entire chromosomes (see below). In the course of the analysis, we noticed that FISH signals against centromeric repeats (CENs) preferentially appeared in the same optical focal plane. Plotting CEN signals along the z -axis relative to the median plane of each nucleus clearly shows a narrow spatial dispersion of FISH signals around a single plane in isolated nuclei, by contrast to their 3D dispersion, preferentially at the periphery, in native endosperm nuclei (Online Resource 1). This observation was

unexpected because in *Arabidopsis* somatic nuclei, CCs (containing centromeres) show an apparent preferential localization at the nuclear periphery (Armstrong et al. 2001; Fransz et al. 2002). In 3D nuclei, CCs follow a distribution with a regular spacing, which can be modeled by introducing a repulsive constraint between CCs (Andrey et al. 2010). Although euchromatin loops emanating from CCs likely explain the regular dispersion of centromeres (de Nooijer et al. 2009; Fransz et al. 2002), they do not intuitively explain a planar alignment in flattened nuclei where one would predict a random distribution. In the light of our observation and former models (de Nooijer et al. 2009; Andrey et al. 2010), it is tempting to propose a hypothetical connective scaffold between centromeres that would favor a regular dispersion in native 3D arrangements while constraining it to a planar alignment in flow-sorted nuclei upon flattening (Online Resource 1).

Centromeres frequently associate in endosperm nuclei

As in somatic tissues (Fransz et al. 2002), endosperm nuclei of *Arabidopsis* harbor heterochromatic CCs, comprising CENs and the 45S ribosomal DNA (rDNA) repeats of the nucleolus organizer regions (NORs) as shown by FISH (Baroux et al. 2007). *Arabidopsis* possess a haploid complement of five chromosomes with two NORs located on chromosomes 2 and 4 (Fransz et al. 2002). In a former FISH study, we noticed that the expected maximal number of CEN signals (15) was rarely seen in triploid endosperm nuclei, suggesting that the chromosomes are usually not completely dissociated.

To gain more insight into the association frequency of CEN and NOR regions in endosperm nuclei, we scored the number of FISH signals corresponding to CEN and 45S rDNA repeats, respectively, in isolated seed nuclei of different ploidy levels. Figure 1 shows the box plot distributions of raw values (left panels) and of values normalized per haploid genome (right panels).

In somatic tissues, rDNA loci were seldom fully separated, a situation explained by their common regulation at interphase (Schubert et al. 2012). Thus, not surprisingly, the number of NOR foci as revealed by FISH rarely (2C, 3C) or never (4C, 6C, 12C) reached the possible maximal number (red dots, Fig. 1a), with two to three foci in 2C, 3C, and 4C nuclei and four to six foci in 6C and 12C nuclei (Fig. 1a, Table 1). By contrast, CEN regions are often separated in somatic nuclei at interphase, although the possible maximal number of ten foci is rarely observed by FISH (Fransz et al. 2002). Starting with a mean number of eight CEN foci in 2C seed nuclei (Table 1), which is similar to that observed in 2C leaf nuclei (Fransz et al. 2002), the mean number of CEN foci increased with higher ploidy, but not in a linear fashion (Table 1, Fig. 1b). If normalized per haploid genome, the number of CEN foci decreased in nuclei with higher ploidy (Fig. 1b). In 4C, 6C

(2n), and 12C (4n) nuclei, which likely correspond to replicated (or endoreduplicated) 2C, 3C, and 6C (4n) nuclei, respectively, this observation may be explained by physical cohesion at the centromeres after replication (Fang and Spector 2005; Schubert et al. 2006). By contrast, 3C (2n) and 6C (4n) endosperm nuclei frequently harbored three CEN foci per haploid genome, with the distributions centered at 3.2 and 3.0, respectively (Fig. 1b). When plotting the association frequency (Fig. 1c), it appeared that centromeres were fully separated in only 1.7 % of the 3C endosperm nuclei ($n = 133$) compared to 17.9 % of the 2C seed nuclei ($n = 54$).

Although homologous versus non-homologous associations were not resolved in these experiments, our observations suggest a very frequent pairwise association at CEN regions.

The number of chromocenters is a function of maternal genome dosage

The triploid endosperm genome consists of two maternal and one paternal chromosomal set (2m:1p). Thus, the question arises whether the pairwise association of CENs involves complements of the same or of different parental origins. While we currently have no means to distinguish the parental origin of CENs using FISH, we made use of endosperm nuclei with imbalanced ratios of parental genomes. We previously described an endosperm-specific interspersed (ESI) heterochromatin fraction, which increases with higher maternal dosage (Baroux et al. 2007). In this former study, we analyzed 2m:2p and 4m:1p endosperm nuclei obtained from interploidy crosses ($2n \times 4n$ and $4n \times 2n$, respectively) and 2m:0p endosperm nuclei that develop in the absence of fertilization and, thus without a paternal contribution, in mutants of the *fertilization-independent seed* (*fis*) class (Baroux et al. 2007). We analyzed this dataset for the number of CCs, identified as bright and regular foci after staining with 4',6-diamidino-2-phenylindole (DAPI). In *Arabidopsis*, up to ten CCs comprise centromeric repeats as well as pericentromeric regions and up to two CCs also comprise NOR repeats (Fransz et al. 2002). Thus, the number of CCs can be taken as a proxy for the number of separate centromeres (compare CEN signals in Table 1 and the CC number in Table 2). We scored maximum intensity projections of 3D image series from nuclei recorded in whole-mount tissues derived from interploidy crosses and *fis* mutants published previously (Baroux et al. 2007). In control nuclei from seed coat and root tissues, we observed a frequency distribution of the number of CCs centering around 8 to 9 (Fig. 1d), with an average of seven to eight CCs (Table 2), respectively, as previously reported for somatic *Arabidopsis* cell types (Andrey et al. 2010; Fransz et al. 2006). As expected, a higher number of CCs were formed in endosperm nuclei (12.4 in average, Table 2); the increase, however, was not proportional to the ploidy level (Fig. 1d). Instead, the parental dosage influenced CC formation.

Fig. 1 Scoring of chromocenters and CEN and NOR FISH signals in seed nuclei with varying ploidy and parental genome dosages. The number of NOR (**a**) and CEN (**b**) FISH signals in isolated nuclei with varying ploidy levels as indicated under the *x-axis*. The box plots show the raw values (*left panel*) and values normalized to the haploid complement (*right panel*). The *red dots* show the maximal expected values of FISH signals (e.g., in 2C nuclei, four NOR and ten CEN FISH signals). The number of nuclei analyzed is indicated *above* the box (*left panel*). Letters (*right panel*) denote groups of significance in post hoc tests using Tukey's HSD multiple comparisons (see [Methods](#)). **c** Frequency distribution of CEN number per nuclei (isolated fractions) and schematic representations of no, full, or pairwise association corresponding to the number of detected CEN signals. **d** Nuclei from different cell types imaged in whole-mount tissues were analyzed for their number of CCs as described (Baroux et al. 2007). Cell types were from somatic diploid tissues (root, seed coat) and from endosperm with wild-type (2m:1p) or altered (2m:2p, 2m:0p, 4m:1p) ratios of parental genomes. *Left panel* raw values; *right panel* values normalized to the maternal dosage

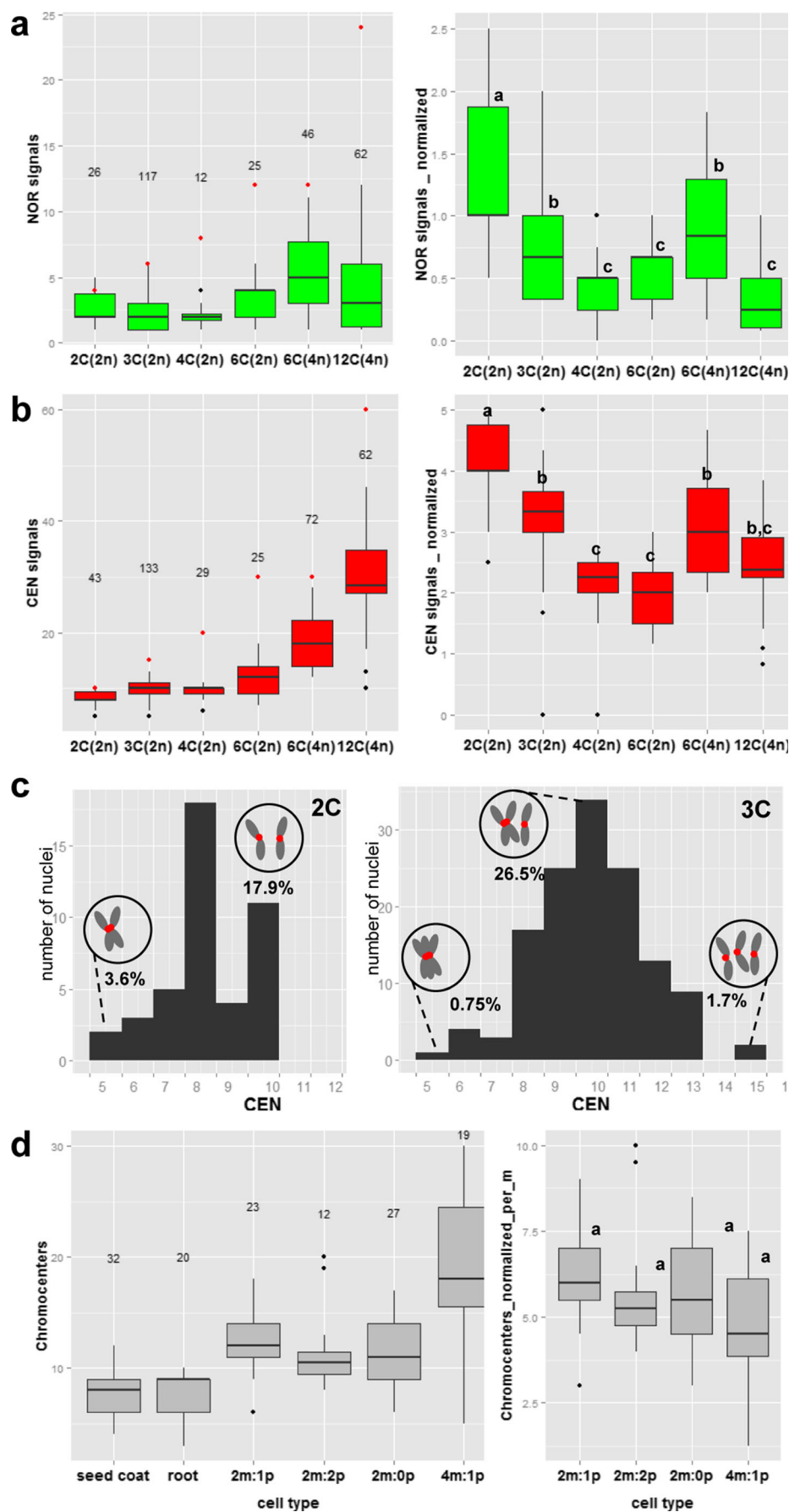


Table 1 Mean volume and number of CEN and NOR foci in ploidy-sorted seed nuclei

	Volume (μm^3)	CEN	NOR
2C	45.4 (± 14.4), $n = 60$	8.2 (± 1.4), $n = 28$	1.7 (± 0.5), $n = 13$
3C	84.8 (± 20.4), $n = 72$	10 (± 1.8), $n = 117$	2.2 (± 1.2), $n = 117$
4C	83.9 (± 16.8), $n = 30$	9.5 (± 1.2), $n = 21$	2.2 (± 1.0), $n = 12$
6C	148.5 (± 78.0), $n = 43$	12 (± 3.0), $n = 25$	3 (± 1.4), $n = 25$
6C (4n)	250.1 (± 87.6), $n = 40$	19.4 (± 4.7), $n = 59$	5.5 (± 2.7), $n = 46$
12C (4n)	374.6 (± 161.5), $n = 20$	30.4 (± 7.4), $n = 62$	3.9 (± 2.9), $n = 62$

Mean (\pm standard deviation), n = number of nuclei, corresponding to the datasets plotted in Online Resource 1a and Fig. 1a–c

Endosperm nuclei carrying two maternal chromosome complements (2m:1p, 2m:2p, 2m:0p) showed distributions with median and mean values ranging from 10 to 12 CCs (Fig. 1d, Table 2) irrespective of the paternal contribution. By contrast, endosperm nuclei with an increased maternal dosage (4m:1p) showed a dramatic increase in CCs with a mean number of 18.7 (Fig. 1d, Table 2). However, when normalizing relative to the maternal dosage, the number of CCs remained largely invariable in the different cell types (Fig. 1d, right panel).

Collectively, these data suggest that the number of CCs is proportional to the maternal dosage and that CCs may preferentially be formed by maternal chromosomes, possibly associated in a pairwise manner, as suggested by our FISH analyses of CENs.

The arrangement of chromosome segments and territories is non-random in endosperm nuclei

To answer the question whether the preferential association of CENs reflects a chromosome-wide organizing principle, we compared the association frequency of CTs in endosperm nuclei to those obtained by a random arrangement computed by a SCD model (Pecinka et al. 2004). The model was adjusted to the mean geometrical dimensions of 3C endosperm nuclei ($n = 100$, Fig. 2a, b). CTs were modeled as chains of domains encompassing ~ 1 Mb, connected by entropic spring potentials. At the start of the model, the chromosomes were considered as

randomly distributed, compressed cylinders, which were then allowed to relax into the ~ 1 -Mb spherical domains (Fig. 2c) as described previously (Pecinka et al. 2004). To compare the outcome of these SCD model simulations with experimental observations, we performed replicate chromosome painting experiments (Pecinka et al. 2004) using probes against the top and bottom arm of chromosome 1 (Fig. 2d). We scored 358 nuclei and reported the frequencies of those with three (fully separated CTs), two (pairwise association), or one (fully associated) CT for each probe set and compared it with the simulated association frequencies (Fig. 2e). A chi-square test revealed a significant difference for both probe sets ($P < 0.01$, chi-square goodness-of-fit test), with an evident underrepresentation of nuclei with fully associated CTs. Increased pairwise association was more evident for the probe set against the bottom arm of chromosome 1 (Fig. 2e, lower graph). Note that chromosomes showing separated chromosome arms occasionally displayed pairwise association at the centromeric regions reminiscent of our FISH results for CENs.

Chromosome painting only grossly assesses the proximity of chromosome arms and provides little information with respect to the actual association of chromosomal segments. Hence, we carried out FISH experiments on 3C nuclei using 80–100-kb genomic probes provided by five bacterial artificial chromosome (BAC) clones (Fig. 2f) as described (Pecinka et al. 2004). We scored the number of nuclei with 1, 2, or 3 FISH signals corresponding to full association, pairwise association, or full dissociation of the three homologous chromosomal segments. The distribution was compared to that obtained from random simulations computed according to the geometrical RSD model (Pecinka et al. 2004), adjusted to 3C nuclei (Fig. 2g). Clearly, all observed distributions deviated from the random expectation ($P < 0.001$, chi-square goodness-of-fit test). Notably, for all probes except MBK21, pairwise associations were found in the majority of the 3C nuclei (44.0–48.6 %, $n = 109$ –182). By contrast, homologous regions encompassing the MBK21 locus were frequently dissociated (57.4 %, $n = 129$).

In summary, these data indicate that pairwise association of chromosomal segments is widespread in 3C endosperm nuclei, but that it may be differently regulated across the genome.

Table 2 Mean number of chromocenters in seed and root tissue, in endosperm nuclei with varying parental dosage

Chromocenters	
Seed coat	7.5 (± 2.0), $n = 32$
Root	7.7 (± 2.3), $n = 20$
2m:1p	12.3 (± 2.7), $n = 23$
2m:2p	11.6 (± 4.0), $n = 12$
2m:0p	11.7 (± 3.5), $n = 27$
4m:1p	18.7 (± 7.0), $n = 19$

Mean (\pm standard deviation), n = number of nuclei, corresponding to the datasets plotted in Fig. 1d

Discussion

The endosperm is a peculiar seed tissue of flowering plants based on developmental, physiological, genetic, epigenetic, and cytogenetic aspects. Endosperm nuclei are triploid, with two maternal and one paternal genome (2m:1p) complement. This imbalanced ratio of parental genomes fosters epigenetic gene regulation, leading to parent-specific gene dosage regulation via genomic imprinting or dosage compensation (Baroux et al. 2002; Birchler 1993; Dilkes and Comai 2004; Köhler and Lafon-Placette 2015). Ultimately, epigenetic regulation influences the development of this embryo-nourishing tissue and hence the outcome of hybridization between plants of different ploidy levels or between distinct species (Birchler 2014; Gutierrez-Marcos et al. 2003; Ishikawa and Kinoshita 2009; Köhler and Kradolfer 2011; Schatlowski and Köhler 2012). The endosperm genome is largely hypomethylated compared to that of the embryo (Gehring et al. 2009), whereby multiple loci are marked by parent-of-origin-specific patterns (reviewed in Li and Berger 2012). Maternal alleles are predominantly hypomethylated in correlation with higher expression levels (reviewed in Köhler and Kradolfer 2011). An interplay between maternal histones modified (H3K27me3) by *Polycomb* Repressive Complex 2 (Bemer and Grossniklaus 2012) and maternally derived small RNAs is thought to reinforce the epigenetic and transcriptional dimorphism between the parental genomes (Köhler and Kradolfer 2011).

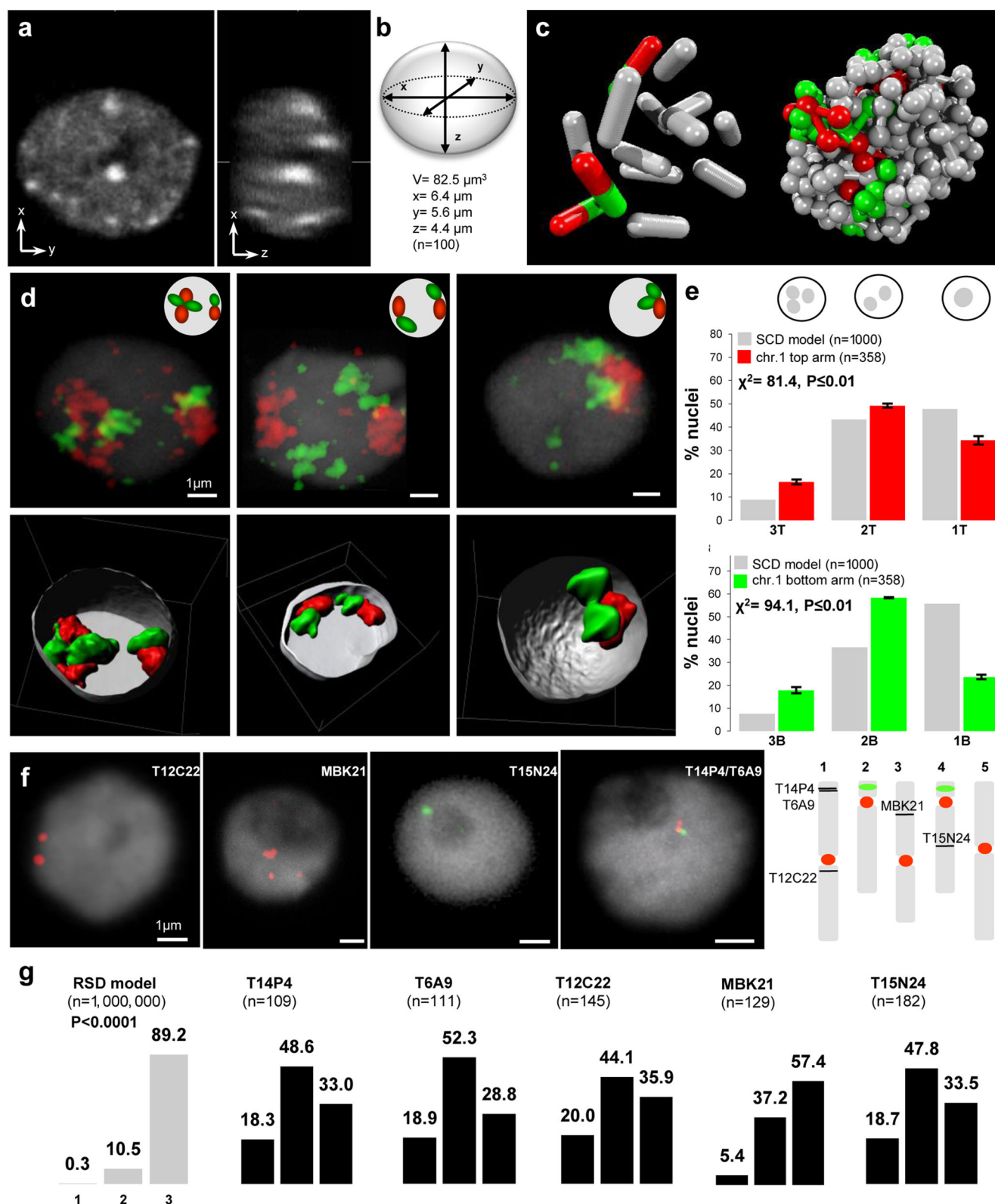
This parent-of-origin-specific epigenetic asymmetry is also detectable in the chromatin of *Arabidopsis* endosperm nuclei by a different histone repertoire (Ingouff et al. 2007). The turnover of sperm-derived H3.3 in the endosperm is passive, getting diluted through successive mitotic divisions. At the microscopic level, paternal chromatin remains physically separated from most or all of the maternal chromatin during the first day after fertilization. In our samples, collected 5–6 days after fertilization, this separation is no longer observed. Previously, we have shown that *Arabidopsis* endosperm nuclei harbor a peculiar chromatin organization that is distinct from that of somatic nuclei. Endosperm chromatin appears less condensed than somatic chromatin and forms smaller CCs but contains ESI heterochromatic foci, which are enriched in H3K9me1 (Baroux et al. 2007), a histone modification of unknown function in plants. Here, we showed that chromosomes adopt a peculiar configuration in endosperm when compared to somatic nuclei: they show a non-random association of centromeres, chromosomal segments, and entire chromosome arms compared to those predicted by simulations of random arrangements. These patterns suggest a frequent pairwise association of two of the three chromosome complements. Although our association frequency analysis focused only on selected chromosome arms and segments (CENs; chromosome 1; five segments on chromosomes 1, 3, and 5), we propose that this non-random chromosome

Fig. 2 Random simulations and measurements of association frequencies of chromosome 1 and five chromosomal segments in endosperm nuclei. **a** *x*, *z*, and *x,y* cross sections of a typical 3C endosperm nucleus isolated by flow-sorting. **b** Mean *x*, *y*, and *z* geometrical dimensions ($n=100$) used for adapting nuclear shells of the RSD and SCD models (Pecinka et al. 2004). **c** SCD model in endosperm nuclei: *left image*, one start configuration featuring all 15 endosperm chromosomes as gray rods with the top and bottom of chromosome 1 colored in red and green, respectively; *right image*, the same nucleus after 1000 relaxations between 1-Mb spherical domains constituting each chromosome (Pecinka et al. 2004). **d** Three representative images of chromosome 1 painting. *Top panel* *x* and *y* views of maximum intensity projections along the *z*-axis; *insets* show the pattern interpreted as scored in experiments. *Bottom panel* 3D reconstructions of serial images and image segmentation for nucleus and chromosome painting signals. **e** Association frequencies of top and bottom arms of chromosome 1 measured in chromosome painting experiments: the histograms show the number of nuclei with 3, 2, or 1 territory for the top (red) and bottom (green) arms and the corresponding expected distribution from the random arrangements predicted in 1000 SCD simulations (gray). *P* values: from chi-square goodness-of-fit tests, $n=358$ nuclei pooled from three replicate experiments. **f** Representative images of FISH experiments using bacterial artificial chromosome (BAC) probes as indicated and corresponding to five distinct chromosomal regions mapping to three of the five *Arabidopsis* chromosomes (*uppermost right picture*). **g** Association frequencies of BAC signals: *left*, gray bars: histogram of random arrangement according to a RSD model. Rest of histograms: the number of nuclei showing one, two, or three signals. The BAC probe and number of nuclei analyzed are shown. All measured distributions show a significant deviation from random ($P<0.001$ in a chi-square goodness-of-fit test). Scale bar = 1 μ m

arrangement is a general feature of *Arabidopsis* endosperm nuclei, although local differences do exist as shown for a segment on chromosome 3 (MBK21), where the three homologs are frequently separated. Whether such local difference coincides with transcriptional activity and/or epigenetic marks remains to be determined.

A frequent chromosomal association has also been reported in endosperm nuclei of wheat, a hexaploid monocot with $2n=6x=42$. In 3C wheat endosperm nuclei, the mean number of centromeric foci is ~ 47 , with more than half of the centromeres being clearly associated (Wegel and Shaw 2005). In wheat, this situation may be explained by the close proximity of chromosomes that adopt a Rabl configuration, which, however, has not been described for *Arabidopsis*. In wheat endosperm nuclei, the Rabl configuration of chromosomes induces the frequent association via their centromeres but does not regularly involve homologs (Wegel and Shaw 2005). Whether the association we observed in *Arabidopsis* endosperm nuclei involves (only) homologs, as we observed it for the arms of chromosome 1, remains to be determined.

Another question arising from our study concerns the parental origin of the chromosomal complements exhibiting pairwise association. Because endosperm nuclei comprise two maternal sets, it is tempting to speculate that maternal homologs associate and occupy a territory that is spatially distinct from the paternal homolog. However, we noticed that the number of CCs depends solely on the maternal genome



dosage with a mean number of ~5 CCs per haploid complement. When the paternal contribution is reduced (2m:0p) or increased (2m:2p), the number of CCs remains equal. This observation indicates that maternal complements are

separated and that paternal and maternal chromosomes associate preferentially. Following interploidy crosses in maize, endosperm nuclei also respond at the level of chromosome organization, with a slightly more pronounced

decondensation of heterochromatic foci (centromeric regions and the knob) upon increased maternal genome dosage (Bauer and Birchler 2006). Whether altered dosage of the parental genome ratio similarly influences the number and association frequency of centromeres and chromosomal arms in a parent-of-origin-specific manner as in *Arabidopsis* remains to be determined.

The question remains as to whether the chromosomal association we observed in *Arabidopsis* endosperm nuclei has any functional significance. Perhaps, the physical proximity enables a coordinated regulation of a pair of parental alleles, while the separated homolog may be amenable to distinct regulation, depending on the dosage sensitivity of the alleles. For instance, dosage compensation of specific alleles encoding storage proteins (e.g., glutenin, zein) and metabolic enzymes (e.g., alcohol dehydrogenase) has been reported in the endosperm of crop plants such as wheat and maize (Birchler et al. 2001; Galili et al. 1986). This phenomenon can hinder the robust overexpression of genes of interest, e.g., for plant molecular pharming (Ou et al. 2014). In addition, the dosage-specific regulation of parental alleles markedly influences the success of interspecific hybridization in flowering plants (Birchler 2014; Lafon-Placette and Köhler 2014; Schatrowski and Köhler 2012). In this respect, it would be interesting to test whether the *SENSITIVE TO DOSAGE IMBALANCE* locus, proposed to buffer the effects of genome dosage imbalance in naturally occurring aneuploids (Henry et al. 2007), influences chromosomal organization in the endosperm.

The observations reported in this study suggest that the triploid genome of *Arabidopsis* endosperm nuclei frequently adopts a configuration where chromosomes are associated in a pairwise manner, possibly involving a complement from each parent (Fig. 3). Future work should address the parental origin of the associated chromosomes. Furthermore, this model sets the stage for future investigations on the extent to which structural chromosome arrangements functionally influence epigenetic regulation in the endosperm, particularly with respect to

genomic imprinting and dosage compensation, the latter still being poorly understood at the mechanistic level.

Methods

Nuclei preparation, FISH, imaging, scoring, and image processing

Seed nuclei were isolated from *A. thaliana* (L.) Heynh accession Landsberg *erecta* as described (Baroux et al. 2007). Chromosome painting and FISH using CEN, NOR, and BAC probes were done as described (Baroux et al. 2007; Pecinka et al. 2004). For scoring FISH signals (Figs. 1 and 2), images were recorded using a Zeiss Axiophot 2 or a Zeiss Axioplan epifluorescent microscope and a Spot 2 (Diagnostic Instrument) or Apogee Alta F (Imaging Systems) cooled charge-coupled digital camera, respectively. Serial optical sections for 3D reconstructions were recorded in confocal laser scanning microscopy with a Leica SP2 (FISH experiments, Figs. 1 and 2, Online Resource 1) or Leica SP5R (live imaging of GFP-CENH3 (Ravi et al. 2011); H1.1-RFP (Baroux et al. 2007) in endosperm nuclei, Fig. 1) instruments (Leica Microsystems). Images from epifluorescence microscopy were processed with Photoshop (Adobe Inc.) for overlaying FISH and DAPI signals (by merging the distinct channels) and moderate Gaussian smoothing. Serial images were processed in Imaris (Bitplane AG) for 3D reconstructions, segmentation of nuclear contours (surface function), and FISH signals (spot function).

Nuclei measurements, FISH signal scoring, and analysis

Nuclear volumes were calculated from manually determined x , y , and z dimensions ($v = \pi xyz / 6$) in cross-sectional projections using Imaris (Bitplane AG). Quantitative scoring of FISH signals was done directly upon microscopic inspection.

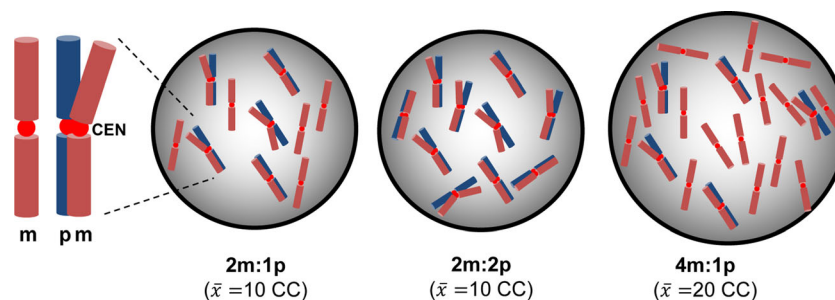


Fig. 3 Model for the pairwise chromosomal association in *Arabidopsis* endosperm nuclei. Based on our observations, we propose a model, in which the $3n$ genome of endosperm nuclei frequently adopts a configuration where chromosomes associate in a pairwise manner. The association is frequent at centromeric regions (hence producing ten signals in FISH with CEN probes), may occur along entire

chromosomes or only partially, and may involve a complement of each parent (m maternal, p paternal). In balanced $2m:1p$ nuclei, the mean number of CCs is 10; upon increased paternal dosage ($2m:2p$), the same number of CCs appears, while the mean number of CCs increases upon increased maternal dosage ($4m:1p$)

Graphics and statistical analyses in Online Resource 1 were computed in R (www.r-project.org). A linear model of the normalized data was generated for ANOVA analysis, and confidence intervals of the differences between means were derived according to Tukey's *honest significant difference* (HSD) method in pairwise comparisons. To analyze the distribution of CEN foci along the *z*-axis (Fig. 1), the *z* position of FISH signals (spot statistics) was expressed as a relative distance from the median symmetric plane orthogonal to the *z*-axis of the nucleus (nucleus dimension and position in the image were retrieved from surface statistics) and plotted in Excel. The distribution of FISH signals according to their class of association (Fig. 1) was compared with random distribution (see below) using a chi-square goodness-of-fit test.

Models to simulate the interphase arrangement of chromosome segments and territories

We used a random spatial distribution (RSD) model for simulating the frequency of possible association between BAC segments (modeled as 100-kb spheres of $0.22 \mu\text{m}^3$) and a spherical chromatin domain (SCD) model for simulating chromosome territories (as a chain of ~1-Mb chromatin domains of 500 nm diameter) as described (Pecinka et al. 2004; Schubert and Shaw 2011). The models were adjusted with the average dimensions of 3C endosperm nuclei ($x=6.46 \mu\text{m}$, $y=5.66 \mu\text{m}$, $z=4.43 \mu\text{m}$, $n=100$). In the RSD model, the results of 1,000,000 nuclei simulations gave the following frequencies of nuclei with 3, 2, and 1 BAC signal, respectively (percentages are in parentheses): 892,361 (89.24 %), 104,727 (10.47 %), and 2912 (0.29 %). The configurations generating one BAC signal include those where the three loci are clustered (three contacts) or aligned (two pairwise contacts). In the SCD model, numerical simulations of 1000 nuclei generated the following distribution of nuclei with fully separated CTs (three signals), pairwise association (two signals), and full association (one signal): 76, 366, and 558 for the top arm of chromosome 1 and 89, 433, and 498 for the bottom arm of chromosome 1.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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